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IMPROVING YIELDS, QUALITY AND BY PRODUCT UTILIZATION OF SASKATCHEWAN-GROWN MILK THISTLE

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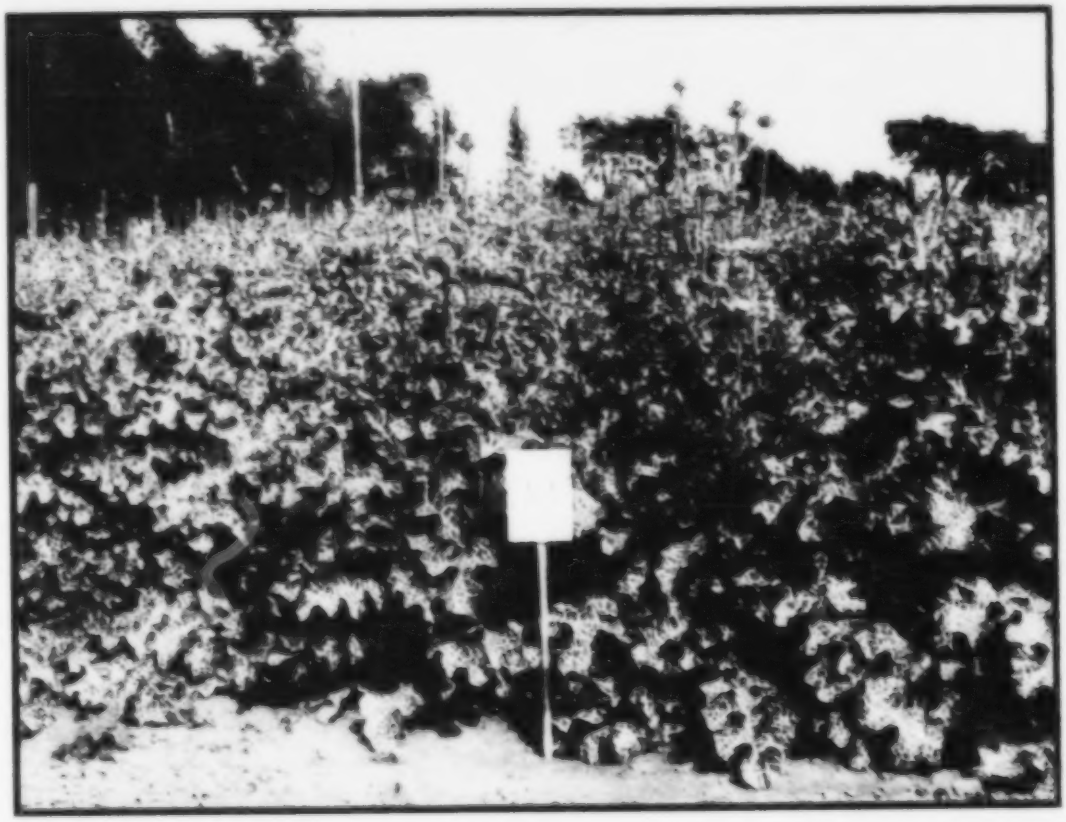

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Abstract

There is worldwide demand for milk thistle (*Silybum marianum*) seed as a herbal medicine. Field trials conducted in 2007 and 2008 sought to develop appropriate agronomic management practices for the improved lines of milk thistle generated by ADF Project No. 20020163. Seed yields from the new shorter stature lines were generally superior to the standard cultivar and the seed also contained higher concentrations of the target phytochemicals. Seed yields and seed quality increased with increasing plant populations to a peak around 100 seeds planted/m2. This yield and quality response may reflect the more uniform flowering and seed development achieved when branching of the milk thistle plants is suppressed by growing in a dense stand. Pre-harvest desiccation was essential to mechanical harvest of the milk thistle crop. Diquat (reglone) and concentrated acetic acid both provided an acceptable degree of desiccation. Diquat is faster acting and less expensive than acetic acid, but acetic acid is perceived more favorably in organic markets. The project also sought to identify potential value in the 99% of the milk thistle plant that is presently discarded. The seed was found to contain about 23% oil with a fatty acid profile comparable to sunflower and corn oil. Assays of the leaves and stems revealed the presence of several compounds of interest in cosmetic, nutra- and /or pharmaceutical applications.

Executive Summary

There is substantial worldwide demand for milk thistle (*Silybum marianum*) seed as a herbal medicine for the treatment of liver disease and certain types of cancer. Agronomy trials conducted in 2007 and 2008 sought to develop appropriate agronomic management practices for the improved lines of milk thistle generated with support from ADF Project No. 20020163. Variables examined in the agronomy trials included ... a) germplasm, b) plant populations and c) method of desiccation. The objective was to develop a suite of agronomic practices that would maximize seed yields and quality in a mechanically harvested milk thistle crop. The new milk thistle lines produced a vigorous stand of relatively short stature plants that were better suited to mechanical harvest than the previously available lines. Seed yields from the new lines were generally superior to the standard cultivar, although the performance of the lines varied from year to year. The seed harvested from the new lines also contained higher concentrations of the target phytochemicals than the standard. Seed yields and seed quality increased with increasing plant populations to a peak at around 100 seed/m2. This yield and quality response to plant population may reflect the more uniform flowering and seed development achieved when branching of the milk thistle plants is suppressed by growing in a dense stand. Pre-harvest desiccation was essential to mechanical harvest of the milk thistle crop. Diquat (reglone) and concentrated acetic acid both provided an acceptable degree of desiccation. Diquat is faster acting and much more affordable than the acetic acid, but acetic acid is perceived more favorably in organic markets.

At present > 99% of the milk thistle plant is discarded as waste. The seed contains about 23% oil – this oil is presently discarded during extraction of the medicinal component (silymarin). The fatty acid profile of the oil is comparable to sunflower and corn oil – but oil yields/unit area are well below the yields obtained from standard oil crops. Milk thistle produces a huge amount of biomass which at present is simply returned to the soil. Assays of the crop residues revealed the presence of several

compounds of interest in cosmetic products and for nutra- and /or pharmaceutical applications. Whether these compounds are of sufficient value and/or are present in commercially viable concentrations in the milk thistle waste products remains to be determined.

Introduction

Milk thistle (*Silybum marianum* (L.) is a flowering plant belonging to the daisy family (Asteraceae). Milk thistle, is known by several other names, including Blessed Milk thistle, Spotted thistle, St. Mary's thistle, Marian thistle, Holy thistle and Variegated thistle. Milk thistle is native to the Mediterranean regions of Europe, North Africa and the Middle East but has been introduced throughout the world. The plant is valued for its medicinal properties, but it is sometimes grown as an ornamental plant because of its unusual leaves. Milk thistle spreads quickly and it is considered a weed in some parts of the world. In the U.S.A., it is found in most southern states and the north-eastern and mid-west states. It has been declared a noxious weed in Washington, Oregon and Texas, but is not considered as a noxious weed in any Canadian province.

Plant Description:
Milk thistle is a vigorous tall upright plant that prefers dry sunny conditions. The spiny stems branch at the top, and reach a height of approximately 1.2 to 2 m. The spiny leaves are wide, with white blotches or veins. Milk thistle gets its name from the thistle like appearance of the leaves and the milky white sap that exudes from any cut surface. Flowers are generally red-purple. A solitary flower develops at the end of each stem. Milk thistle has an indeterminate growth and flowering habit, resulting in uneven development and maturity of flower heads. The mature fruit (achene) is relatively small with an attached white silky pappus. In the literature, the achenes, i.e. the 'fruit' are mostly (wrongly) referred to as 'seed'. The immature fruit is soft and cream, tan, or brown in colour and the mature fruit is hard-skinned, shiny brown or brown with tan spots. Due to the indeterminent flowering habit at any point in time the milk thistle plant has some flowers that are still opening while others have progressed to the point where the seed is shattering. To address this issue of uneven seed maturity the mature heads are usually gathered by selective hand harvest. It should be emphasized that the large thorns on the stems, leaves and seed heads of milk thistle make hand harvest of the seed heads an exceedingly unpleasant task. While once-over machine harvest is clearly preferable from the perspective of efficiency, seed yield and quality may be compromised.

In the relatively cool and short Saskatchewan growing conditions, milk thistle is grown as an annual while in warmer longer growing environments milk thistle can be grown as a biennial.

Uses of Milk Thistle:
Every part of milk thistle including the stems, leaves, flower buds are edible. Milk thistle has been used for over 2,000 years as a herbal remedy for a variety of ailments, particularly for liver and gall bladder problems. Studies suggest that substances in milk thistle (especially a flavonoid called silymarin) protect the liver from toxins, including certain drugs such as acetaminophen (Tylenol), which can cause liver damage in high doses. Silymarin has antioxidant and anti-inflammatory properties, and it may help the

liver repair itself by growing new cells.

The leaves are used as a salad green or cooked. Leaves can be trimmed of prickles and boiled or added raw to salads. There are as yet no known medicinal properties for milk thistle leaves. It is claimed that in recent times a number of herbal medicine manufacturers have introduced products containing milk thistle leaves but these products had no apparent therapeutic value.

Constituents:
The principal extract of milk thistle fruit, silymarin (4% to 6% in ripe fruit), is composed of several polyphenolic flavonolignans. The major component (60%) is silybin (also known as silibinin or silybinin) is also the most biologically active component. Other components include silichristin (also known as silychristin, silycristine or silicristin), a metabolic stimulant, and silydianin. Silymarin is found in highest concentrations in the fruit of the plant. Other constituents are flavonoids, a fixed oil (16% to 18%), betaine, trimethylglycine (TMG) and amines.

Study Description:

There is substantial worldwide demand for milk thistle (*Silybum marianum*) seed as a herbal medicine. Both 'conventionally' and 'organically' grown milk thistle are sought by the market place. Previous ADF-supported Research (Project No. 20020163) conducted by the University of Saskatchewan identified superior high yielding locally adapted germplasm suited for mechanical harvest under Saskatchewan growing conditions. The Saskatchewan Herb and Spice Association has been granted exclusive rights to grow the high yielding, high quality milk thistle lines developed in ADF 20020163. This agreement insures that the benefits of the previous research project will flow to growers working in collaboration the SHSA.

Previous research conducted at the Canada-Saskatchewan irrigation Diversification Centre demonstrated that there is excellent potential for mechanized production of high quality milk thistle in Saskatchewan even using the relatively unsuitable cultivars previously available (c/o Richters seed). One of the main objectives of the present project is to develop agronomic refinements needed to maximize yields and quality of the new milk thistle lines selected at the University of Saskatchewan.

Saskatchewan's relatively short growing season combined with uneven maturity of milk thistle is a major challenge for production, particularly if the objective is to harvest the crop via once-over combining. By increasing the plant population in the field it may be possible to reduce branching, thereby better synchronizing flowering, resulting in more uniform crop maturity. More uniform maturity should facilitate machine harvest while also reducing shattering loss and improving the overall quality of the harvested seed.

Due to its robust growth habit and indeterminent flowering pattern the milk thistle crop has to be desiccated prior to harvest. Previous research conducted at the CSIDC and University of Saskatchewan has demonstrated that Reglone (diquat) is an effective desiccant for milk thistle. Reglone is presently registered under regulatory review for use in Canada as a milk thistle desiccant. However, the market preference for organic milk thistle necessitates the identification of organic desiccants for top-kill.

At present, only a small component of the milk thistle plant (silymarin from the seed) has

market value. The seed contains significant qualities of oil - yet this oil is presently regarded as a waste product. The plant also produces a huge amount of biomass - another waste product unless alternate uses are discovered. Potential uses of the waste biomass are animal fodder or alternatively a starting point for biofuel - especially as the stems and leaves are rich in high energy latex compounds. These latex compounds may also have value as a feedstock in the manufacture of industrial materials ... like rubber and latex. At present, there are no crops being grown for latex in Canada - but there is demand for latex as the starting point in the manufacture of rubber and other materials. The potential to extract products of potential use as fuel, food, fodder and medicine from a single easy to grow plant would appear to make milk thistle an ideal model for of a future bio-based economy.

This project : (i) Investigated agronomic practices designed to further increase yields and quality of selected lines of milk thistle in Saskatchewan, and (ii) Examined the effectiveness of vinegar as an organic desiccant, and (iii) Sought to identify potential value in the "waste products" generated by the milk thistle crop.

All the milk thistle lines being utilized in this project are under exclusive licence to the Saskatchewan Herb and Spice Association - therefore, all line specific information generated in this project will be of direct and exclusive benefit to Saskachewan growers of this crop.

1.0 Agronomy Section

Field trials were conducted during the summers of 2007 and 2008 at the Canada-Saskatchewan Irrigation Diversification Centre, Outlook. Six milk thistle cultivars, (U of S-1, U of S-2, U of S-3, U of S-4, Lone Wolf, and Richters) were evaluated in 2007. Due to the poor performance of the Lone Wolf selection in 2007, this selection was omitted from the 2008 trials. Five seeding rates (25, 50, 100, 125, and 150 seeds/m^2), two desiccants (Reglone (conventional) and Vinegar (organic) were evaluated for each cultivar separately. Field plots were laid out on Split-plot Design with four replications. Main-plot consisted of Desiccants and the Sub-plot consisted of seeding rates. Individual plots (Sub-plots) were 3.7 m x 1.2 m.

The soil at the test site during the three years was a clay loam. In the spring the land was prepared in the traditional manner to form a firm seed bed. Plots were seeded using a double-disc press drill (Appendix Plate 1) with seeds placed at approximately 1-2 cm depth. Rows were spaced 60 cm apart with appropriate seeding rates as defined by the treatments. The crop was raised under dryland growing conditions, as previous experience at CSIDC showed that irrigation of milk thistle prolonged vegetative growth and delayed maturity, resulting in considerable yield loss. The only exception was that two light irrigations were applied after seeding to ensure germination and proper crop establishment. One weeding during early growth stage was sufficient to raise the crop. The crop was desiccated (Appendix Plate 2) using Reglone and Vinegar when approximately 50% of the flower heads had matured i.e. formed of pappus on the fruit. The application rate consisted of 2.7 l/ha of Reglone applied in 1000 l water/ha during 2007 and 2008, and vinegar, (14% acetic acid at 1000 l/ha in 2007, and 60% acetic acid at 1000 l/ha in 2008). The crop was harvested once the stalks were sufficiently dry (Appendix Plate 3) to pass through a Wintersteiger plot combine (Appendix Plate 4).

Silymarin content of the harvested seed was analysed by Phytovox Inc., Edmonton, Alberta. Pooled samples from all replicates of the lowest (25 seeds/m^2) and highest (150 seeds/m^2) seeding rates, the two desiccation treatments (Reglone and Vinegar) for each of the different milk thistle lines were analysed for Taxifolin, Silychristin, Silybin A, and Silybin B.

Trial Details:

2007.
Land was prepared in the conventional manner to produce a suitable seed bed. Test plots were seeded on May 18, 2007 using a Fabro seeder. Two light irrigations were applied after seeding to ensure germination and proper crop establishment. The crop was otherwise grown under dryland conditions, based on previous experience with milk thistle.

The 2007 growing season was relatively warm with July being exceptionally warm and dry. The crop received 229 mm of rain (Figure 1.1). Hail storms on July 31, 2007 and August 18, 2007 caused considerable damage to flower heads, negatively affected seed filling and resulting in substantial yield losses. The crop was desiccated on August 14 with Reglone and vinegar at appropriate concentrations. The crop growth/harvest stages and crop desiccation are shown in Appendix Plates 5-11. Seed was harvested on August 30, 2007 and 31, 2007 using a Wintersteiger plot combine. The harvested seed was dried at 35°C. The seed was cleaned and seed yield was recorded.

2008.
Land preparation was done in the conventional manner to produce a suitable seed bed. Spring soil analysis at the test site indicated 71 kg NO_3-N/ha, 59 kg P/ha, 370 kg K/ha, and >86 SO_4-S/ha at 0-30 cm depth. The test plots were seeded on May 14, 2008. Two light irrigation were given soon after planting to ensure germination and proper crop establishment. Subsequently, the crop was grown under dryland condition. Flowering occurred around July 21 to July 28 and the crop was ready for desiccation three to four weeks later. The crop was desiccated on August 22 & 23.

NB – whereas in previous trials **12-14% non-synthetic or "natural" acetic acid** was used as the organic desiccant, in 2008 **60% (synthetic) acetic acid was used**. The concentrated acetic acid was applied at the same rate as used in 2007 (1000 l/ha). **It should be noted that the synthetic acetic acid is not on the list of materials approved for use within "organic production systems.**

Both desiccation treatments were ready for combining within a week of treatment (August 29, 2008).

The growing season was relatively warm and received 131 mm of rain during the crop growth period. Hail storm on August 21, 2008 caused slight damage to the crop.

Results and Discussion

Growing Season Climatic Conditions:
The 2007 and 2008 growing season temperatures were relatively similar, with July being fairly warm (Figure 1.1). The crop received 229 mm of rain in 2007 and 137 mm in 2008 (Figure 1.2).

In 2007, hail storms on July 31 and August 18 caused considerable damage to flower heads that negatively affected seed filling resulting in substantial yield losses, but not complete crop failure. No hail was experienced in 2008.

The various growth stages of milk thistle are illustrated in Plates 5 to 8, stages of flower development in Plate 9, and fruit (i.e. seed) development pappus formation in Plate 10. In this discussion, the term 'seed' will be used to describe the actual fruit, i.e. the achene. Consequently, 'seed yield' signifies the yield of the mature achenes.

Seed Yield:
In 2007, the two hail events caused early death of the crop and also caused significant amounts of shattering of seed from the seed heads. In 2008, despite lower rainfall seed yields were substantially higher than 2007. This confirms the fact that inclement weather during the latter stages of the crop, particularly after maturity, can cause considerable crop losses.

The effects of desiccation methods and seeding rate on seed yield during 2007 and 2008 for the various milk thistle selections are summarized in Table 1.1 and Table 1.2 respectively. The corresponding effects of treatments on average seed weight are summarized in Table 1.3 and Table 1.4 respectively.

Cultivar Response:
In 2007, the selection U of S-2 produced the highest average seed yield of 200 kg/ha, while the cultivar from Lone Wolf produced the lowest yield of 13.4 kg/ha (Table 1.1). The yield ranking was U of S-2 > U of S-4 > U of S-1 > Richters > U of S-3 > Lone Wolf.

In 2008, the average seed yield for the different selections ranged from as low as 756 kg/ha for U of S-1 up to 946 kg/ha for U of S-4 (Table 1.2). The yield ranking was U of S-4 > U of S-3 > Richters > U of S-2 > U of S-1. These seed yields are comparable to yields obtained in previous "successful" years (see ADF project No 20020163).

Seeding Rate Effects:
Seeding rate significantly affected seed yield in both 2007 and 2008 (Tables 1.1 and 1.2). Yield responses to seeding rate were variable for the different milk thistle selections during the two years. The mathematical relationships between seed yield and seeding rate during 2007 and 2008 for U of S-1, U of S-2, U of S-3, U of S-4, and Richters are presented in Figs 1.3-1.7 respectively. The responses were variable for U of S-1, U of S-2, and U of S-3 between the two years. For example, the seed yield for these selections increased in a linear manner with increasing seeding rate in 2007 and assumed a quadratic relationship in 2008. For U of S-4 and Richters, the seeding rate response was quadratic in both years. However, it is not clear why the quadratic responses were so dissimilar over the two years for the Richters line of milk thistle

(Figure 1.7).

The optimum seeding rate for the various cultivars can be estimated from the vertex of the yield response curve (Table 1.3). The optimum seeding rate for the various milk thistle lines was somewhat variable but it appears that a seeding rate between 100 to 125 seeds per m^2 tended to produce the highest seed yields

The two desiccation methods used, i.e. Reglone or Vinegar, provided an equivalent degree of crop desiccation and had no effect on seed yield in 2007 and 2008 (Tables 1.1 and 1.2). In 2007, the crop had been severely damaged by hail prior to application of either top-killing treatment. This hail damage may have rendered the crop susceptible to even the relatively mild effects of desiccating with relatively dilute (12%) acetic acid.

In previous trials (ADF project No 20020163) the Reglone treatment had consistently produced higher seed yields than the organic desiccant. This yield advantage was attributed to the much more rapid desiccation achieved with Reglone – as a rapid drydown would reduce the time during which the mature seed could shatter out. In the 2008 trial the drydown achieved with the "organic" desiccant was as quick and thorough as that achieved with the Reglone. This may be atrributed to (i) the change over to more adapted, earlier maturing milk thistle lines, (ii) use of production practices conducive to acclerated crop maturity (ie; reduced fertility, minimal irrigation, high seeding rates) and c) the use of much more concentrated acetic acid (60%) in 2008 versus 12% acetic acid in 2007.

Average Seed Weight:
Seeding rates and desiccation methods had no effect on seed weight for any of the milk thistle lines tested in 2007 and 2008. Average seed weight (g/1000 seed) for the different selections varied between 16.5 to 18.4 g in 2007 (Table 1.4) and from 14.7 to 22.2 g in 2008 (Table 1.5). U of S 3 produced the smallest seed (14.5 g/1000 seed); U of S 2, U of S 4, and Richters produced larger seeds (20.5 - 22.2 g/1000 seed), and U of S 1 produced intermediate seed (16.5 g/1000 seed).

Quality (Flavonoids):
The flavonoid profile for the various milk thistle selections was influenced by seeding rates and desiccation methods. These results are summarized in Table 1.6 (individual values) and Table 1.7 (treatment averages).

This preliminary analysis showed the following trends with respect to the effects of treatments on flavonoid for the various milk thistle selections:

- seeds from U of S 2 at 150 seeds/m^2 desiccated with Reglone and U of S 1 at 25 seeds/m^2 also desiccated with Reglone contained the highest and lowest amounts of all the flavonoids respectively. The total flavonoid levels ranged between 11.48 and 21.66 µg/g (i.e. 1.2% and 2.2%) of seed dry weight.

- U of S 2 contained the highest amounts of Taxifolin, Silychristin, Silybin A, Silybin B, and Total flavonoids relative to the other selections. In previous trials U of S 2 had consistently produced superior levels of the various bioactive molecules (see ADF project No 20020163).

- seeds harvested from plots with the highest plant population (150 seeds/m^2) had higher flavonoid levels than seed from plots with the lowest plant population (25 seeds/m^2). This may reflect the earlier and more uniform seed maturity achieved with a higher plant population. The flavonoid content of milk thistle seed is know to increase with seed maturity, although it peaks and stabilizes well before the seed is ready to drop from the seed head. The relative amounts of the different flavonoids are also known to change with seed maturity, however these changes are also strongly influenced by genotype, growing conditions and method of extraction and analysis. There is still some considerable debate within the industry as to which of the flavonoid components is the best indicator of "potency" of the milk thistle extracts. Until this debate is settled, the overall silymarin content is considered to be the "default" indicator of quality.

- there was no difference in flavonoid levels between the two desiccation treatment (Reglone or Vinegar).

Conclusions

The objective of the agronomy trials conducted in this project was to develop appropriate management practices for the improved lines of milk thistle generated with support from ADF Project No. 20020163. These trials built on the fertility recommendations and planting practices generated as part of ADF Project No. 20020163. Variables examined in the agronomy trials included ... a) germplasm, b) plant populations and c) method of desiccation. The new milk thistle lines produced a vigorous stand of relatively short stature evenly maturing plants that were better suited to mechanical harvest than the line previously used as the industry standard (c/o Richters). Seed yields from the new lines were generally superior to the standard cultivar, although the performance of all lines varied from year to year. The nature of this genoype X environment interaction needs to be further explored in order to increase confidence in the performance of a specific line(s). The seed harvested from the new lines also contained higher concentrations of the target phytochemicals than the standard. The combination of improved yields and superior seed quality indicates that the new lines released from Project No. 20020163 are meeting expectations. In particular, the line UofS-2 has emerged as providing a combination of superior seed yields and seed quality in a vigorous but readily managed short stature, fast maturing plant phenotype.

Seed yields and seed quality increased with increasing plant populations to a peak at around 100 seed/m2. This yield and quality response to plant population may reflect the more uniform flowering and seed development achieved when branching of the milk thistle plants is suppressed by growing in a dense stand. The dense stand also reduced weed competition early in crop development. While the heavy seeding rate would increase seeding costs, the corresponding yield and quality advantage is clearly sufficient to offset this cost.

This study, as well as ADF Project No. 20020163 showed that there was a significant year X genotype interaction for both the total concentration and the relative amount of different bioactive flavonoids found in the milk thistle seed extract. It also appeared that the flavonoid content and composition could be influenced by plant populations but was not influenced by the method of desiccation. These interactions complicate the process of identifying and adopting genotypes and production practices that optimize product

quality. This process is further complicated by the fact that the industry has not been able to identify which of the flavonoid components is the best indicator of "potency" of the milk thistle extracts. Until this debate is settled, the overall silymarin content should likely be considered to be the "default" indicator of quality. This project has identified genotypes and production practices designed to maximize both yields and product quality.

While the new short stature, even maturing milk thistle lines are clearly better suited to mechanical harvesting than the previous standard lines, pre-harvest desiccation is still essential to efficient mechanical harvest of the milk thistle crop. Diquat (reglone) and concentrated acetic acid both provided an acceptable degree of desiccation. It should be noted that thorough coverage is essential for effective kill down of a vigorous milk thistle crop – and this coverage hinges on the application of very large volumes of the desiccant (1000L/a). Growers must be cautioned against trying to scrimp on the desiccant volume – otherwise the top-kill process will be compromised. Of the two desiccants tested, Diquat is more readily available, faster acting and much affordable. Registration of reglone for use in milk thistle is undergoing regulatory review in Canada. The main reason that a grower would consider use of acetic acid is demand in the organic marketplace. This raises a potential problem. In previous research (ADF Project No. 20020163) the naturally sourced 12% acetic acid provided a slow and incomplete degree of top kill even at very high rates of application. While this product provided an acceptable degree of top kill in the 2007 crop it should be noted that this crop had been weakened by a severe hail event and was therefore relatively easy to kill down. For this reason it was decided to test the efficacy of a much more concentrated form (60%) of acetic acid in 2008. This formulation provided the very fast and thorough desiccation required for consistent machine harvest of milk thistle. However, it should be noted that this form of acetic acid is derived from a synthetic process that uses petrochemicals as starting materials. This effectively disqualifies this product from approval for use in "certified organic" production. In discussions with the SHSA, the issue of which product a milk thistle crop is desiccated with has not raised any particular concern. The market is not presently requiring organic certification and is not paying a premium for organically grown milk thistle. This would suggest that in the present market situation desiccation with either Reglone or concentrated acetic acid are viable options.

Table 1.1. Seeding rate and desiccant effects on seed yield for milk thistle line selections: 2007 trial

Treatment	Seed yield (kg/ha)					
	U of S-1	U of S-2	U of S-3	U of S-4	Lone Wolf	Richters
Desiccant:						
Reglone	158.1	184.9	74.6	183.0	14.4	107.7
Vinegar	162.7	214.8	68.9	201.4	12.4	118.7
Seeding rate:						
25 seeds/m^2	110.1	99.4	47.8	138.9	3.5	58.8
50 seeds/m^2	158.4	144.3	70.3	194.3	7.6	100.1
100 seeds/m^2	157.5	219.3	73.1	244.8	18.1	130.7
125 seeds/m^2	172.3	249.8	91.8	171.6	20.6	132.6
150 seeds/m^2	203.7	286.5	75.7	211.3	17.3	143.9
ANOVA						
Source:						
Desiccant	ns	ns	ns	ns	ns	ns
Seeding rate	**(64.9)	***(69.8)	*(38.4)	ns	**(12.6)	***(51.4)
Desiccant x Seeding rate	ns	ns	ns	ns	ns	ns
C.V. (%)	27.7	24.0	36.6	40.7	64.5	31.1

***, **, *, and ns indicate significance at P<0.001, 0.01, 0.05 levels of significance and not significant respectively. Values within parentheses are LSD estimates at 5.0% level of significance.

Table 1.2. Seeding rate and desiccant effects on seed yield for milk thistle line selections: 2008 trial

Treatment	Seed yield (kg/ha)				
	U of S-1	U of S-2	U of S-3	U of S-4	Richters
Desiccant:					
Reglone	738.8	789.0	898.2	1029.4	871.7
Vinegar	773.4	801.1	841.2	862.6	849.8
Seeding rate:					
25 seeds/m2	651.9	627.8	684.3	741.5	846.4
50	855.3	722.4	860.4	915.2	835.8
100	756.7	853.4	935.2	1097.5	833.1
125	813.0	920.8	917.7	937.5	858.7
150	703.5	850.7	951.0	1038.4	929.9
Analyses of variance					
Source					
Desiccant (D)	ns	ns	ns	ns	ns
Seeding rate (R)	*(137.4)	***(117.7)	**(120.1)	*(240.9)	ns
D x R	ns	Ns	ns	ns	ns
C.V. (%)	17.6	14.3	13.4	24.7	22.5

***, **, *, and ns indicate significance at P< 0.001, 0.01, 0.05 levels of probability and not significant respectively. Values within parentheses are LSD estimates 5.0% level of significance.

Table 1.3. Summary of the mathematical relationships between seeding rate and seed yield of milk thistle line selections: 2007 and 2008

Selection	Year	Response	R^2	Vertex
U of S-1	2007	Linear	0.82	-
U of S-1	2008	Quadratic	0.52	89
U of S-2	2007	Linear	0.99	-
U of S-2	2008	Quadratic	0.96	125
U of S-3	2007	Linear	0.62	-
U of S-3	2008	Quadratic	0.92	121
U of S-4	2007	Quadratic	0.55	102
U of S-4	2008	Quadratic	0.78	113
Richters	2007	Quadratic	0.97	139
Richters	2008	Quadratic	0.96	-

Table 1.4. Seeding rate and desiccant effects on average seed weight for milk thistle line selections: 2007 trial

Treatment	1000 Seed weight (g)					
	U of S-1	U of S-2	U of S-3	U of S-4	Lone Wolf	Richters
Desiccant:						
Reglone	18.0	17.5	18.5	16.0	16.7	17.0
Vinegar	18.2	18.2	18.2	17.6	16.8	16.0
Seeding rate:						
25 seeds/m^2	18.9	16.6	17.7	18.2	15.1	14.6
50 seeds/m^2	17.8	17.4	18.4	16.5	18.7	16.6
100 seeds/m^2	18.0	17.6	18.0	17.4	17.8	16.8
125 seeds/m^2	18.0	19.0	18.6	14.4	16.0	17.4
150 seeds/m^2	17.9	18.7	19.0	17.6	16.2	17.2
ANOVA						
Source:						
Desiccant	ns	ns	ns	ns	ns	ns
Seeding rate	ns	ns	ns	ns	ns	*(2.7)
Desiccant x Seeding rate	ns	ns	ns	ns	ns	ns
C.V. (%)	6.7	13.7	9.9	18.6	24.0	11.2

* and ns indicate significance at P< 0.05 level of significance and not significant respectively. Value within parenthesis is LSD estimate at 5.0% level of significance.

Table 1.5. Desiccation and seeding rate effects on average seed weight of milk thistle line selections: 2008 trial

Treatment	1000 seed weight (g)				
	U of S-1	U of S-2	U of S-3	U of S-4	Richters
Desiccant:					
Reglone	16.7	22.0	14.7	20.5	21.1
Vinegar	16.2	22.4	14.3	20.4	21.0
Seeding rate:					
25 seeds/m^2	16.7	22.4	13.5	20.4	21.5
50 seeds/m^2	16.8	21.6	15.0	21.1	20.9
100 seeds/m^2	16.1	22.1	14.7	20.5	21.2
125 seeds/m^2	16.0	22.6	14.2	20.0	20.9
150 seeds/m^2	16.7	22.4	15.1	20.2	20.7
Analyses of variance					
Source					
Desiccant (D)	ns	ns	ns	ns	ns
Seeding rate (R)	ns	ns	ns	ns	ns
D x R	ns	ns	ns	ns	ns
C.V. (%)	10.9	5.1	2.0	4.8	8.0

ns indicate non significant treatment effects.

Table 1.6. Effects of seeding rate and desiccation on seed flavonoid content in milk thistle line selections.

Selection	Desiccation	Seeding rate (seeds/m^2)	µg per mg dry weight				
			Taxifolin	Silychristin	Silybin A	Silybin B	Total
U of S - 1	Reglone	25	1.371	3.896	2.661	3.549	11.48
U of S - 1	Reglone	150	1.747	4.782	3.407	4.484	14.42
U of S - 1	Vinegar	25	2.102	5.066	3.525	4.669	15.36
U of S - 1	Vinegar	150	2.115	5.747	4.097	5.367	17.33
U of S - 2	Reglone	25	2.496	6.078	4.551	5.834	18.96
U of S - 2	Reglone	150	2.998	6.984	5.081	6.593	21.66
U of S - 2	Vinegar	25	2.514	5.732	4.204	5.484	17.63
U of S - 2	Vinegar	150	2.746	6.451	4.769	6.189	20.15
U of S - 3	Reglone	25	1.562	4.319	2.987	3.921	12.79
U of S - 3	Reglone	150	1.747	4.692	3.222	4.369	14.03
U of S - 3	Vinegar	25	1.591	4.668	3.279	4.304	13.84
U of S - 3	Vinegar	150	1.863	5.366	3.811	4.962	16.00
U of S - 4	Reglone	25	1.405	5.354	3.852	5.123	15.73
U of S - 4	Reglone	150	1.454	4.98	3.548	4.644	14.63
U of S - 4	Vinegar	25	1.199	4.378	3.099	4.156	12.83
U of S - 4	Vinegar	150	1.586	5.476	3.892	5.182	16.14
Richters	Reglone	25	1.876	4.895	3.423	4.528	14.72
Richters	Reglone	150	2.204	5.176	3.557	4.703	15.64
Richters	Vinegar	25	1.178	4.558	3.234	4.26	13.77
Richters	Vinegar	150	1.956	4.350	2.960	3.948	13.21

Table 1.7. Treatment effects on average flavonoid levels in seed of milk thistle line selections.

Treatment	µg per mg dry weight				
	Taxifolin	Silychristin	Silybin A	Silybin B	Total
Selection:					
U of S-1	1.834	4.873	3.423	4.517	14.65
U of S-2	2.689	6.311	4.651	6.025	19.6
U of S-3	1.691	4.761	3.325	4.389	14.17
U of S-4	1.411	5.047	3.598	4.776	14.83
Richters	1.939	4.745	3.294	4.36	14.34
Seeding Rate:					
25 seeds/m^2	1.783	4.894	3.482	4.583	14.71
150 seeds/m^2	2.042	5.4	3.834	5.044	16.32
Desiccant:					
Reglone	1.886	5.116	3.629	4.775	15.41
Vinegar	1.939	5.179	3.687	4.852	15.63

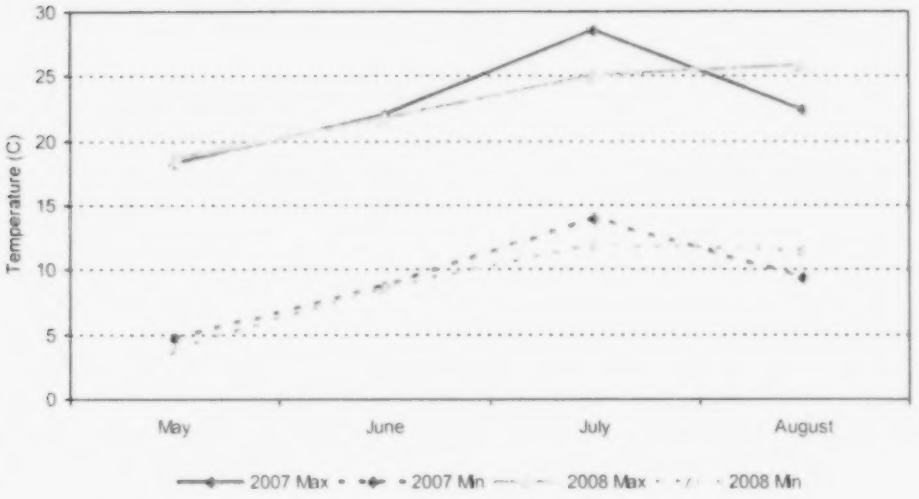


Figure 1.1. Monthly average maximum and minimum temperature during the milk thistle growing season: 2007 and 2008.

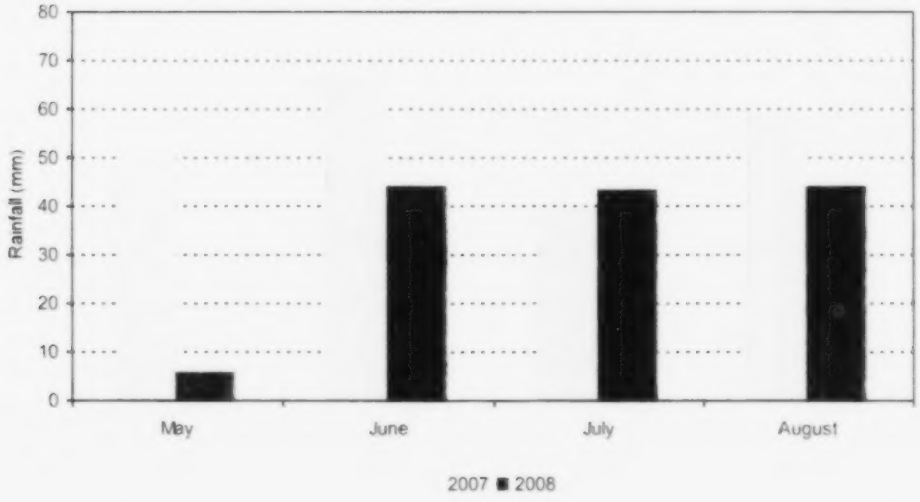


Figure 1.2. Monthly rainfall distribution during the milk thistle growing season: 2007 and 2008.

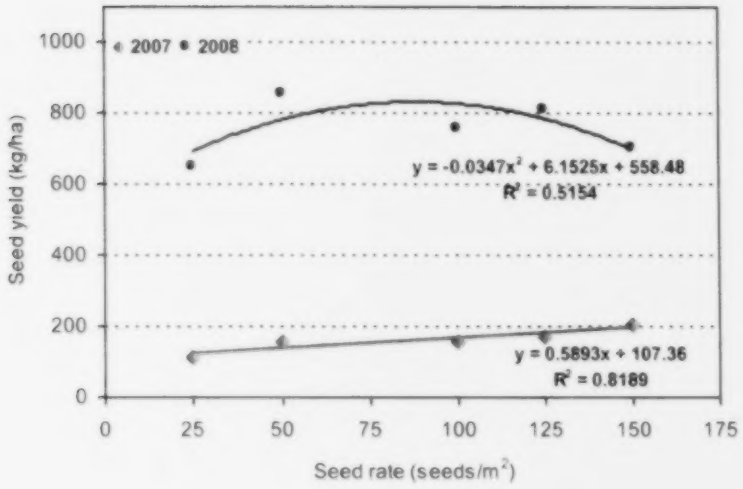


Figure 1.3. Relationship between seeding rate and seed yield for the milk thistle line UofS-1 grown under dryland conditions in 2007 and 2008.

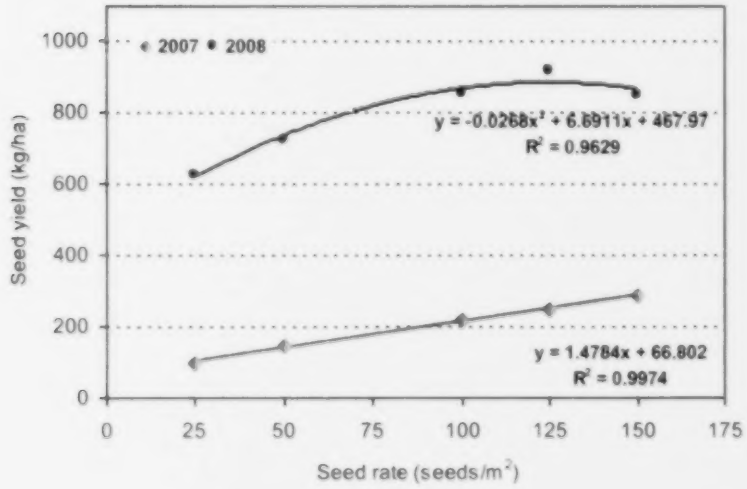


Figure 1.4. Relationship between seeding rate and seed yield for the milk thistle line UofS-2 grown under dryland conditions in 2007 and 2008.

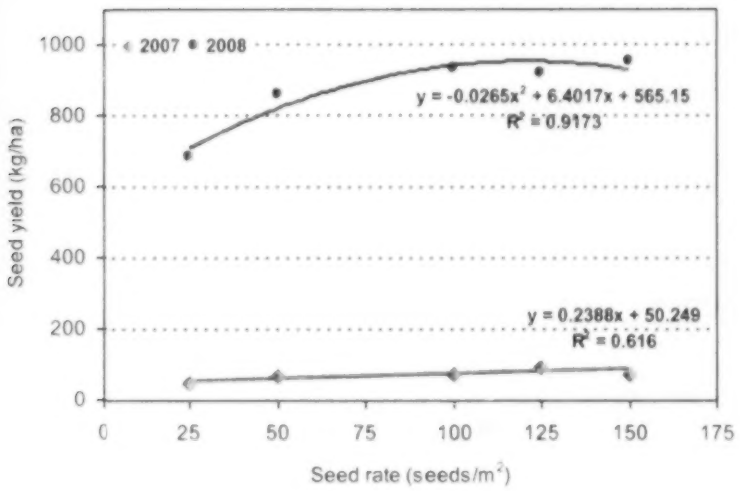


Figure 1.5. Relationship between seeding rate and seed yield for the milk thistle line UofS-3 grown under dryland conditions in 2007 and 2008.

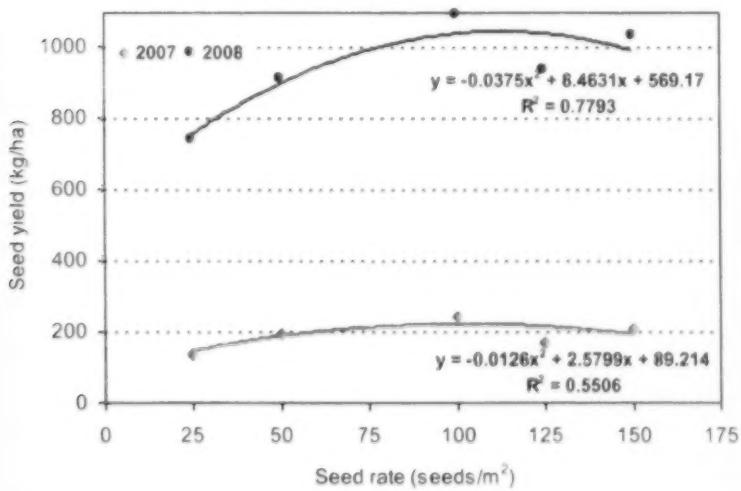


Figure 1.6. Relationship between seeding rate and seed yield for the millk thistle line UofS-4 grown under dryland conditions in 2007 and 2008.

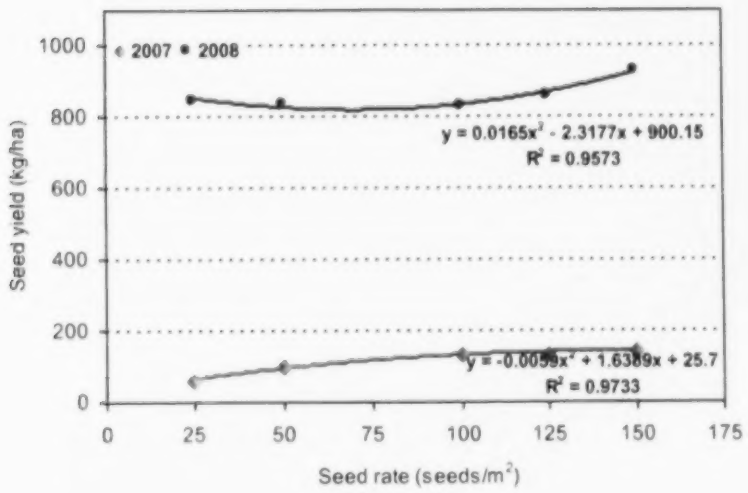


Figure 1.7. Relationship between seeding rate and seed yield for the Richters milk thistle line grown under dryland conditions in 2007 and 2008.

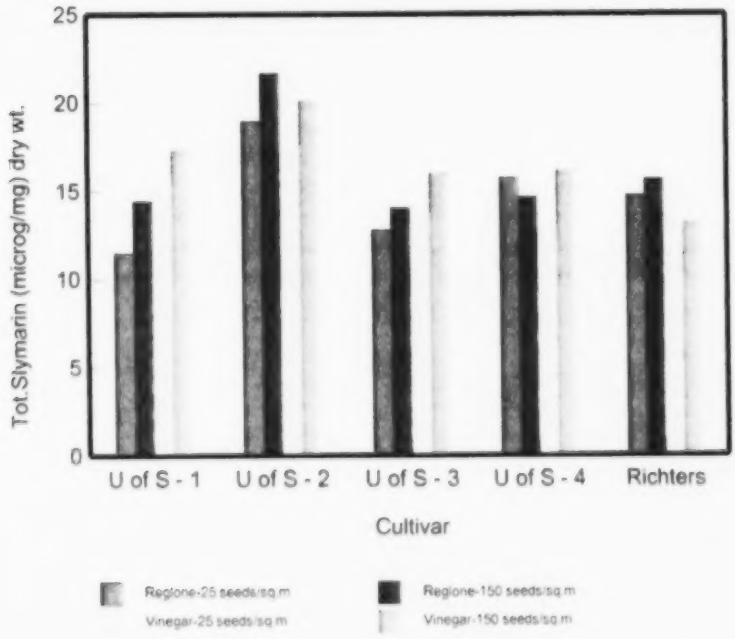


Figure 1.8. Seeding rate and desiccation effects on total Silymarin content in mature seed of milk thistle selections.

2.0 Recovery of Value-added Products from Milk Thistle

2.1. Seed oil

The research literature indicates that milk thistle seed contains 12-26% fixed oils, depending on the genotype and the growing conditions. At present, these oils represent a waste product which is discarded following silymarin extraction from the seed.

This project evaluated the oil concentration, composition, total yield and potential value of the oils recovered from the newly developed milk thistle lines being commercialized by the SHSA.

Experimental design

Seed source - SHSA lines generated in agronomy trials

Variables examined

- Total oil content
- Oil composition
- Impact of agronomic variables and site/growing season on oil yields

Milk Thistle seed samples covering the previously outlined range of variables (cultivar, site of production, year of production) were sent to Dr. Martin Reaney of the Dept. of Applied Microbiology and Food at the University of Saskatchewan for analysis.

Oil was extracted from small samples of milk thistle seed using a Goldfisch extraction apparatus and the oil composition was than tested via NMR. A cold press was used to extract the oil from larger (1 kg) samples of a limited number of seed lots.

Averaged over the cultivars tested, seed oil content was lower in 2004 (20.2%), than in 2005 (25.2%), or 2006 (24.9%)(Table 2.1). The low oil content in 2004 likely reflects the immature stage of the crop when hit by an early killing frost. The 2005 and 2006 growing seasons were much more favorable, leading to higher yields of more mature seed with a higher seed oil content. Differences in seed coil content between cultivars were not consistent from year to year. Oil yields from cold press extraction were comparable to the yields obtained using the Goldfisch apparatus (Table 2.1).

Table 2.1. Seed oil content for various milk thistle lines in 2004-2006.

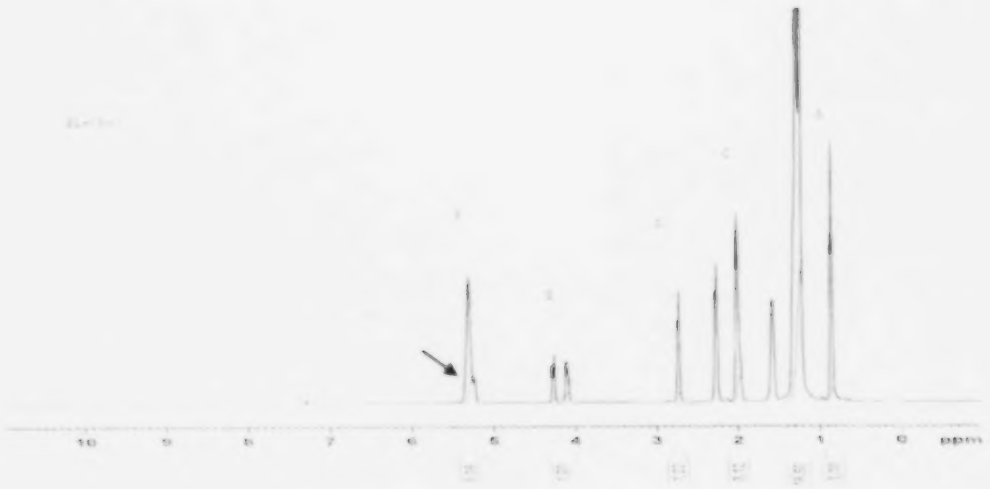
Sample ID	% Oil Content
Richter's (2004)	20.77
Austra Hort. Holland (2004)	18.44
Kalyx Holland (2004)	24.55
Bolier Holland (2004)	22.58
Cruz. E. Europe (2004)	17.3
Richter's (2005)	25.21
Austra Hort. Holland (2005)	25.01
Kalyx Holland (2005)	24.94
Bolier Holland (2005)	25.86
Austra Hort. (2006)	23.73
Bolier (2006)	26.18
Austra Hort. (2004) 1kg	20.53
Bolier Holland (2004) 1Kg	22.67
Kalyx Holland (2005) 1Kg)	26.81

NMR analysis of the oils showed no significant differences among the samples (data not shown). The fatty acid profile of all samples was essentially similar (Table 2.2).

Table 2.2 Fatty acid profile of the oil extracted from the seed harvested from several cultivars of milk thistle in 2004-2006.

	A	B	C	D	E	F	G=E/4 G	F1=F-G F1
Richter's 2004	3	16.605	3.12	1.22	1.2016	3.0485	0.3004	2.7481
Austra Hort. Holland 2004	3	16.819	3.0887	1.2034	1.2161	3.005	0.304025	2.700975
Kalyx Holland 2004	3	16.953	3.1906	1.253	1.2543	3.118	0.313575	2.804425
Bolier Holland 2004	3	16.64	3.0944	1.2187	1.2262	3.0275	0.30655	2.72095
Cruz E. 2004	3	16.8	3.0829	1.2015	1.2063	2.9927	0.301575	2.691125
Austra Hort. Holland 2005	3	17.195	3.1331	1.1815	1.2405	2.9865	0.310125	2.676375
Richter's 2005	3	17.129	3.0706	1.1557	1.202	2.9553	0.3005	2.6548
Kalyx Holland 2005	3	17.021	3.0466	1.1346	1.1892	2.9125	0.2973	2.6152
Austra Hort. 2006	3	16.733	3.0806	1.2159	1.2098	3.3093	0.30245	3.00685
Austra Hort. 2004 1Kg	3	16.664	3.0597	1.1389	1.1585	2.9165	0.289625	2.626875
Bolier 2006	3	17.409	3.0263	1.038	1.2072	2.8339	0.3018	2.5321
Bolier Holland 2005	3	17.279	2.9565	1.0042	1.1806	2.7318	0.29515	2.43665

Fig 2.1. NMR analysis of the oil profile typical of Milk Thistle.



The seed oil content and fatty acid profile of milk thistle oil, along with several of the most commercially important oils used in food production are summarized in Table 2.3.

Table 2.3. Seed oil content (%) and fatty acid profile (%) of oil extracted from Milk Thistle seed and several other commercially important oil crops.

	Seed oil content	Unsat/sat ratio	Palmitic (16:0)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)
Milk Thistle	**23**	**6:1**	**9**	**5**	**23**	**57**	
Canola	43	15:1	4	2	61	21	10
Sunflower	47	7:1	7	5	19	68	
Corn	4	7:1	11	2	24	57	
Soybean	18	6:1	11	4	24	57	7

The fatty acid profile of the oil extracted from milk thistle was comparable to the oil extracted from corn, sunflower and soybean. This suggests that the oil would likely have acceptable performance characteristics for use in food processing – assuming that it does not have any unusual/undesirable flavour characteristics. However, the fact that the oil composition is comparable to mainstream commercial products like corn and soybean oil means that it will be in direct market competition with these low cost alternatives.

Based on the seed yields and seed oil content seen in this study, oil production/unit area for milk thistle is well below that of the key oil producing crops (Table 2.4). The low oil yield/unit area, coupled with the fact the fatty acid profile is not unique suggests that growing milk thistle as an oil crop is not an attractive option. However the results do suggest the potential to use the milk thistle oil that is left over form silymarin extraction as another profit stream – much like the wine industry is capturing value (grape seed oil) from the residues left from their main processing objective.

Table 2.4. Oil production/unit area for milk thistle and other oil producing crops

	Seed Yield (#/a)	Seed Oil (%)	Oil Yield (#/a)
Milk Thistle	**1000**	**23**	**230**
Canola	1500	43	645
Sunflower	1100	47	517
Corn	6700	4	260
Soybean	2500	18	450

2.2 Identification/Recovery of other useful by-products from milk thistle

Milk thistle gets its name from the "milky" exudate that oozes from any cuts to the leaves, stems or roots. The chemical composition of the milk thistle exudate is not known - but closely related species have been identified as sources of latex. The fact that milk thistle produces a large biomass with minimal management or inputs, suggests its potential efficiency as a 'bio-factory'. As the vast majority of the milk thistle plant is, at present, effectively a waste product - any alternate uses for this waste material would represent another potential profit source.

Test material grown in 2006 and 2007 by the University of Saskatchewan was made available to Dr. John Balsevitch, G. Bishop and L. Deibert – who are natural products biochemists employed by PBI/NRC, Saskatoon.

Leaves of milk thistle were harvested just past full flowering. The leaves were allowed to air dry and processed *via* grinding and followed by extraction with 60% methanol (aq) followed by 100% methanol. A 50 g sample of dried leaf material yielded after evaporation of the combined methanolic extracts about 15 g of residue.

The combined methanolic extract was analyzed by HPLC-MS and observed to contain two main flavonoid glycuronides tentatively identified as apigenin and luteolin 7-O-glucuronides (Fig 2.2.1) based on the spectral data and a published report of similar compounds being observed in the flowers.[1]

In the samples examined the putative "apigenin" was the major flavonoid (ca. 7:3 ratio).

Processing of the extract *via* partitioning between ethyl acetate/water and butanol/ water afforded three fractions – ethyl acetate soluble (1.1 g), butanol soluble (1.8 g) and water soluble (12 g). The ethyl acetate fraction was composed of lipophilic materials including

pigments and triterpenes, the butanol fraction was composed largely of the flavonoid glucuronides, while the aqueous fraction contained polar materials believed to be unidentified salts and amino acids/proteins. Samples of relatively pure flavonoids could be obtained by further processing the flavonoid containing extracts on a reverse phase column using gradient water-methanol elution.

2.2.1 Flavonoids in Milk Thistle

In general, flavonoids are widespread in the plant kingdom but glycuronide derivatives are not the most common representatives – glycosides and aglycones are much more common. Flavonoids are mostly anti-oxidants, considered to possess nutraceutical qualities, and by and large, generally recognized as safe (GRAS status). Apigenin glucuronides have previously been isolated from alfalfa leaves [2] and galacturonides identified in milk thistle flowers [1].

Searching the patent literature afforded two [3,4] patents which describe utilization of flavonoid glycuronides. In one they were observed to promote improved solubility and uptake of various drugs; in the other they were used to prolong the life of natural anthocyanin pigments used as coloring agents.

1 R = H, Apigenin 7-O-ß-D Glucuronide, MW 446

2 R = OH,Luteolin 7-O-ß-D Glucuronide, MW 462

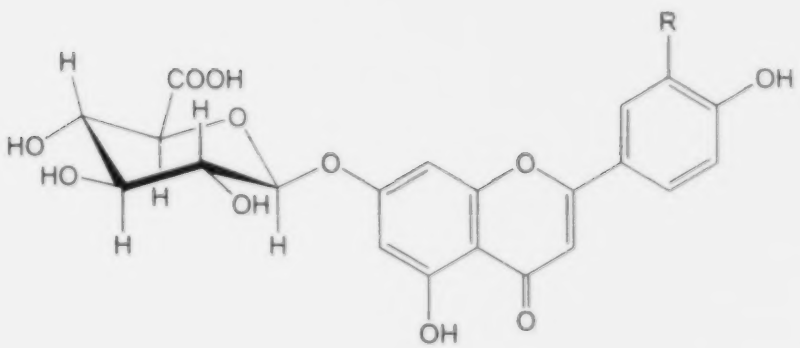


Fig. 2.2.1. Putative structures of major (1) and minor (2) flavonoids observed in air-dried mature *Silybum marianum* leaves obtained from field plants, University of Saskachewan 2006/07.

2.2.2 Non-Flavonoid Components

Examination of the hplc-ms data (see Appendix 2.1) led to the tentative identification of triterpenes having a molecular weight of 456, possibly ursolic and oleanolic acids. Previously reported[5] oxygenated triterpenes were not observed. The leaves are also considered a rich source of amino acids (both free and in proteins, (14%) as well as magnesium and potassium (source: http://www.ars-grin.gov/cgi-bin/duke/farmacy2.pl) and are considered edible on removal of the spiny thorns. The bulk of the extract was the water soluble material which, based on the above report, was likely a mixture of salts, amino acids, proteins, and possibly polysaccharides.

2.2.3 Bioassays/Reported Activities

The major flavonoid (MW 446) was evaluated in hemolysis and apoptosis assays. It was found not to be hemolytic in sheep red blood cells (HD_{50} >100 µM) and did not cause any increase in caspase 3/7 activity or mitochondrial pertabation in PC-3 human prostate cancer cells at 50 µM (Dual Sensor MitoCasp assay using flow cytometry). Both of these assays suggest that the flavonoids have low cytotoxicity. This is further corroborated by historical usage of the leaves as a tea:

"The flowers and leaves of the milk thistle plant can also be used in an infusion. A milk thistle hot tea is used to stimulate milk production in nursing mothers, as well as to treat digestive problems." (http://www.disability-resource.com/medical-health/alternative-medicine/herbal/antioxidant-rich-milk-thistle-herbal-remedy.php). The above results in combination with reports of historical usage suggest low toxicity of the contained flavonoids.

Leaf extracts have been shown to extert anti-inflammatory properties in rats.[7]

2.2.4. Conclusion: Potential Utility of Aerial Parts of Plant

Since the aerial part of the plant represents quite a large amount of biomass, suitable processing could lead to quantities of triterpenes (ursolic and oleanolic acids), potentially useful in cosmetic formulations (based on published anti-inflammatory properties), apigenin/luteolin glucuronides potentially useful in nutra- and /or pharmaceutical applications as outlined in the cited patents, and protein/amino acids potentially useful for food/feed applications.

For commercial development, nutraceutical claims for the flavonoids would have to be established and identification of salts and amino acids present in the aqueous fraction would be required.

Finally, development of milk thistle without thorns would be interesting – perhaps the plant could then be developed as a nutritious salad crop for human use or as a forage crop. Allergy considerations may temper potential food uses.

3.0 Project Related Extension Activities

- the CSIDC milk thistle agronomy trials were toured on several occasions in 2007 and 2008.
- the results were presented at the Saskatchewan Herb and Spice Association Annual Conferences in 2007 and 2008.

Financial support from Saskatchewan Agriculture and Food was acknowledged in all presentations related to this project

We anticipate generating one or two peer reviewed publications from this study.

4.0 Budget

The budget was revised in Dec 2008 at the request of CSIDC and approved by SDAF

	Budgeted	Actual
Salaries	**$ 28,500**	**$ 29,386**
External Services	**$ 2,500**	**$ 2,033**
Rentals	**$ 4,000**	**$ 4,000**
Materials	**$ 2,500**	**$ 2,081**
Total	**$ 37,500**	**$ 37,500**

The Office of Research Services at the University of Saskatchewan has provided a detailed breakdown of the expenditures.

References

1. AA Ahmed, TJ Marry, SA Matlint. 1988. Flavonoids of the flowers of *Silybum marianum*. *Phytochemistry* 28: 1751-53.
2. A Stochmal, S Piacente, C Pizza, F De Riccardis, R Leitz, W Oleszek. 2001. Alfalfa (*Medicago sativa* L.) Flavonoids. 1. Apigenin and Luteolin Glycosides from Aerial Parts. *J. Agric. Food Chem.* 49: 753-758.
3. US Patent 7,119,075 Oct 10, 2006. European Patent EP1526860
   Plant based agents as bioavailability / bioefficacy enhancers for drugs and nutraceuticals. Qazi, Ghulam Nabi (Jammu, IN, US) Bedi, Kasturi Lal (Jammu, IN, US) Johri, Rakesh Kamal (Jammu, IN, US) Sharma, Subhash Chander (Jammu, IN, US) Tikoo, Manoj Kumar (Jammu, IN, US) Tikoo, Ashok Kumar (Jammu, IN, US) Abdullah, Sheikh Tasaduq (Jammu, IN, US) Singh, Kuldip (Jammu, IN, US) Pandita, Rashmi (Jammu, IN, US) Suri, Om Parkash (Jammu, IN, US) Gupta, Bishan Datt (Jammu, IN, US) Suri, Krishan Avtar (Jammu, IN, US) Satti, Naresh (Jammu, IN, US)
   Abstract: The invention relates to the isolation and preparation of an active fraction from plant *Cuminum cyminum*, its further purification and standardization as chemically defined entity (ies) with their intended use as drug bioavailability enhancer for the drugs belonging to therapeutic categories such as antimicrobial, antifungal, anti-viral, antitubercular, antileprosy, anti-inflammatory, antiarthritic, cardiovascular, antihistaminics, respiratory distress relieving drugs, immunosuppressants, anti-ulcerogenic, anti-cancer, CNS drugs, corticosteroids, nutraceuticals in compositions to be administered orally/parenterally, topically, inhalations (including nebulizers), rectally, vaginally in human beings and/or veterinary conditions.
4. US patent 5,908,650. June 1, 1999. Lenoble; Rod (Westminster, CO), Richheimer; Steven L. (Westminster, CO), Bank; Virginia R. (Boulder, CO), Bailey; David T. (Boulder, CO)
   Abstract: An improved pigment composition containing an anthocyanin pigment and a pigment-improving agent selected from the group consisting of flavonoid glycuronides, flavonoid glucuronides, and caffeic acid derivatives. The pigment-improving agents deepen and improve the intensity of the anthocyanin pigment and increase its stability in the presence of light, heat and/or pH.
5. E Ahmed, A Noor, A Malik, S Ferheen, N Afza. 2007. Structural determination of silymins A and B, new pentacyclic triterpenes from *Silybum marianum*, by 1D and 2D NMR spectroscopy. *Magnetic Resonance in Chemistry* 45: 79-81.
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Appendix 1. Agronomy Trial for Milk Thistle

Plate 1. Fabro seeder for milk thistle.

Plate 2. Chemical desiccation of milk thistle.

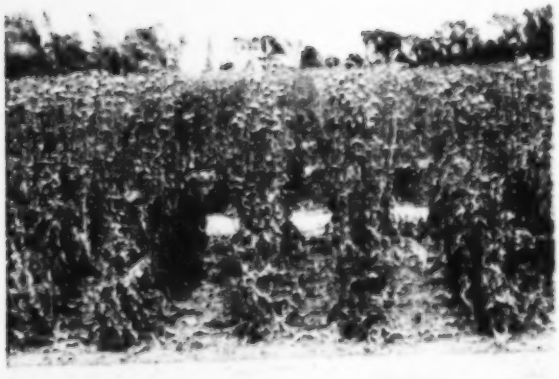


Plate 3. Milk thistle ready for combining.

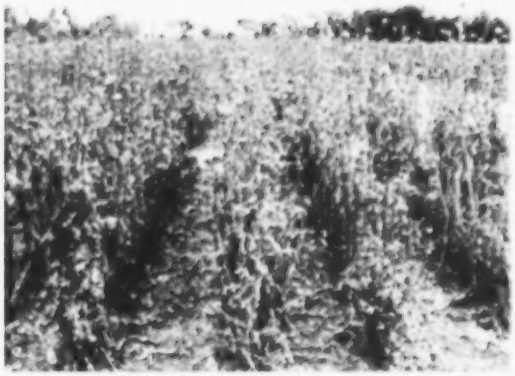
Plate 4. Combining milk thistle.

Plate 5. Milk thistle crop in Late June.

Plate 6. Milk thistle test plots in mid July.

Plate 7. Milk thistle at flowering stage

Plate 8. Milk thistle flower.



Plates 9 and 10. Milk thistle crop desiccated with Reglone (late August)

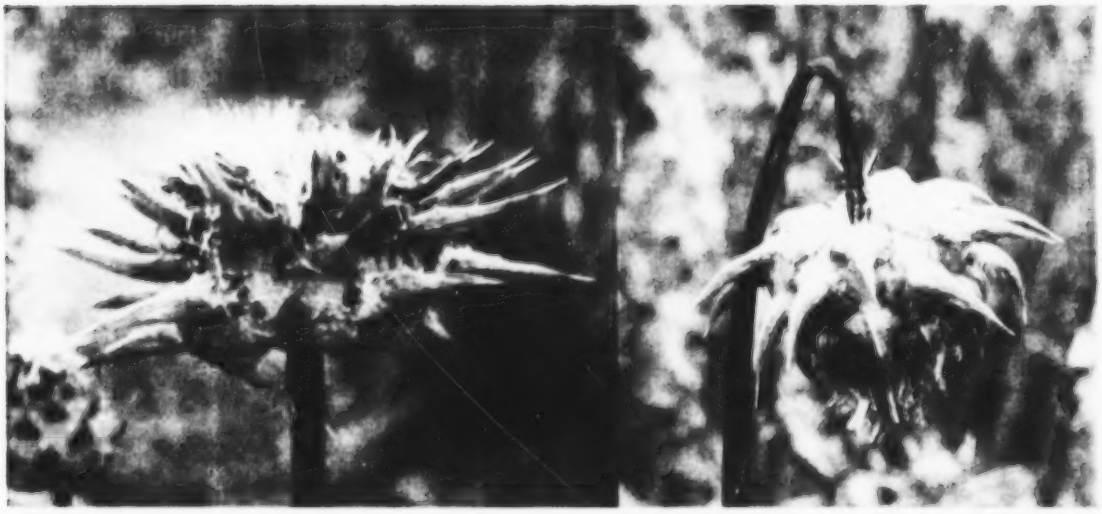


Plate 11. Desiccated milk thistle flower head ready for combining (late August).

Appendix: 2.1 HPLC-MS Data for Milk Thistle Leaf Extracts

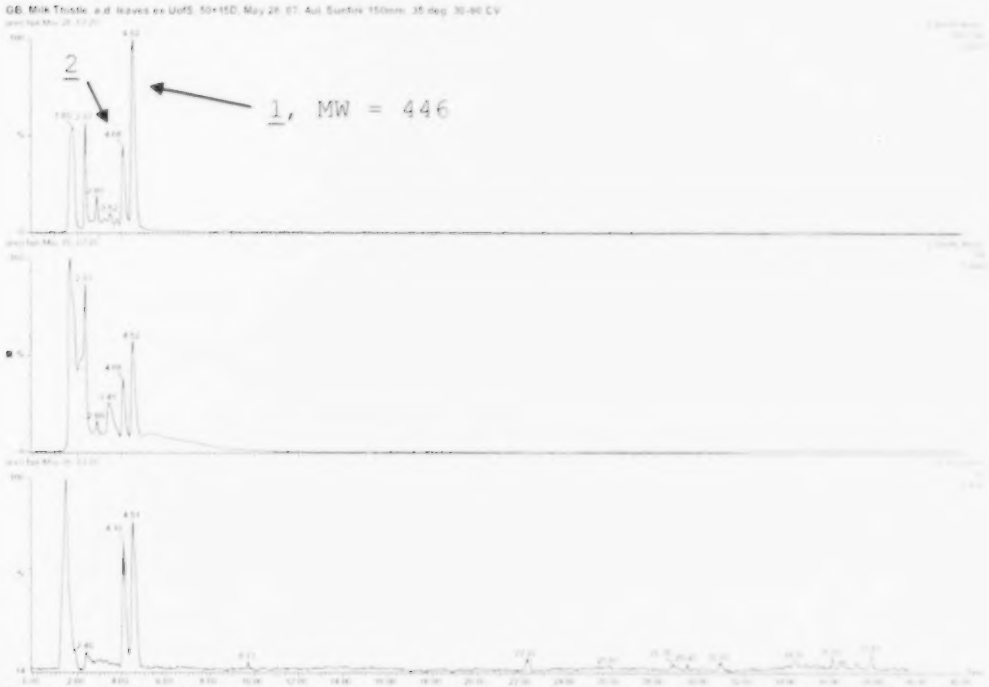


Fig. 2.2.1. HPLC profiles of extract obtained from air-dried mature leaves of milk thistle. Top: uv detection @ 280-340 nm; Middle: uv detection @ 200-400nm; Bottom: mass detection (*m/e* 100 – 1900).

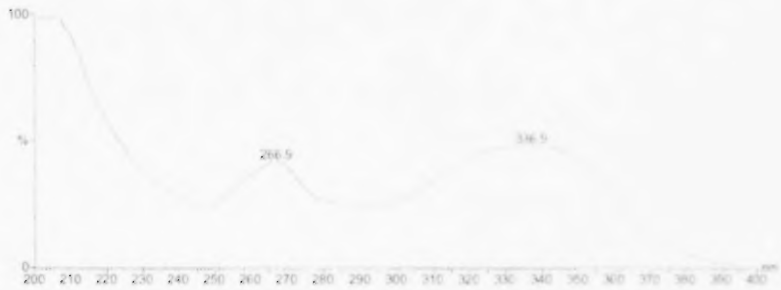


Fig 2.2.2. UV spectrum of compound 1 and 2.

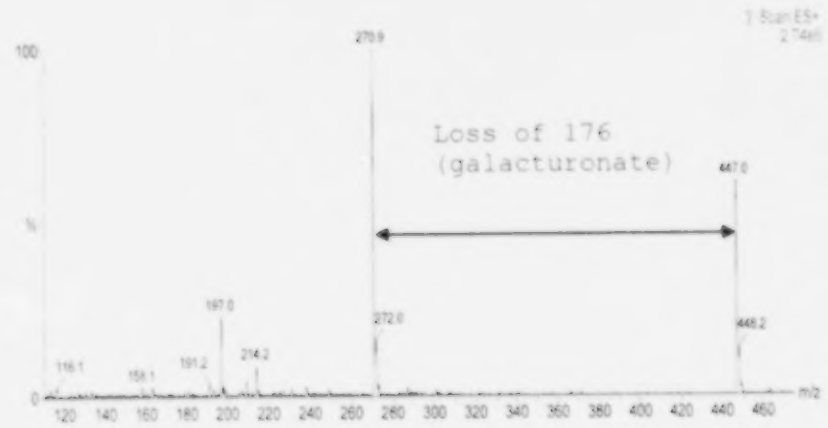


Fig. 2.2.3. Mass spectrum (electrospray, positive ion) of compound 1, showing loss of galacturonate (or glucuronate).

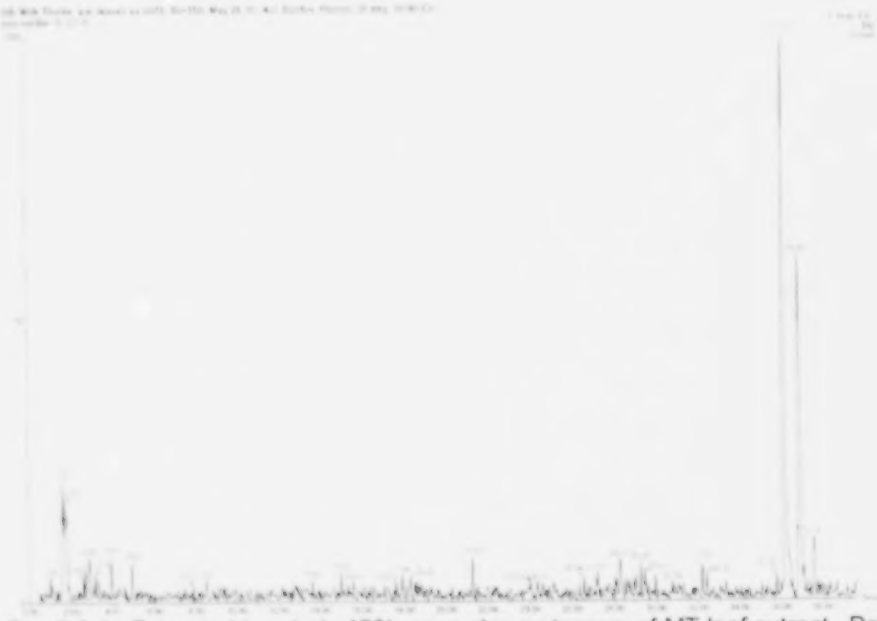


Fig. 2.2.4. Extracted ion (m/z 456) mass chromatogram of MT leaf extract. Peaks tentatively assigned as oleanolic and ursolic acids.